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(54) Title: PROCESS FOR IMMOBILIZATION OF BIOLOGICAL MATERIAL BY CROSS-LINKING WITH POLY-AZETIDINE (57) Abstract Immobilization method comprising mixing with polyazetidine, dewatering, sub-dividing and curing. The method is useful for enzymes, cell mass, coenzymes and antibodies. The product is physically strong, and less cross-linking agent can be used than in prior-art processes.		

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Process for immobilization of biological material by cross-linking with polyazetidine.

This invention relates to a process for immobilizing biological materials by cross-linking with polyazetidine.

5 BACKGROUND OF THE INVENTION

The field of immobilized biologically active materials emerged about twenty years ago, and since then a large number of publications have appeared on immobilization techniques and on the uses of immobilized products. Such
10 studies cover immobilization of enzymes, cells, antibodies and co-enzymes.

Several uses of immobilized products have been commercialized in this period, ranging from the use of glucose isomerase in large industrial plants to the use of immo-
15 bilized antibodies for purification purposes.

Among the many immobilization methods known in the art, one group of methods is known as cross-linking, as described in K. Mosbach, Methods of Enzymology, 44 (1976). Cross-linking occurs by reacting the material with bi- or
20 polyfunctional cross-linking agents.

Glutaraldehyde has been used as a cross-linking agent both for enzymes (see e.g. DK 143569) and for microbial cells (see e.g. US patent 3,779,869). Yet, glutaraldehyde is not an ideal cross-linking agent, as it only reacts with -NH_2
25 and -SH groups. Many enzymes and cells react poorly with glutaraldehyde due to a low content of these groups.

A polyazetidine polymer may be employed advantageously for cross-linking purposes, as disclosed in European patent publication EP 0 206 687. The polyazetidine poly-

mer cross-linking system is more widely applicable to immobilization than is glutaraldehyde because it reacts with -COOH, -OH and -NH-groups, as well as -NH₂ and -SH groups.

The immobilization method disclosed in EP 0 206 687 5 is, however, not ideal in the following respects:

- The pasty consistency of the cell mass at the time of mixing with the polyazetidine polymer makes the mixing process difficult.
- The amount of cross-linking agents is fairly large, generally 5-30% of polyazetidine and 5-40% of glutaraldehyde, and preferably 10-20% of each (% of cell sludge dry matter). We believe that large amounts become necessary because of poor mixing with polyazetidine due to the pasty consistency at the time of mixing.
- 15 - The resulting particles are very dense with high diffusion limitation, probably due to the big amount of cross-linking agents.

Immobilization with polyazetidine is also described in G.J. Calton et al., Biotechnology, vol. 4, pp. 317-320 (1986), in US patent 4,436,813, in Wood et al., Bio/Technology, December 1984, pp. 1081-1084, and in European patent publication EP 0 089 165. However, these are not pure cross-linking methods, but instead involve attachment of active cells to a carrier. Carrier bound immobilization is in many cases undesirable as the use of a carrier dilutes the activity leading to low-activity products, and an expensive carrier material is often required.

OBJECT OF THE INVENTION

It is an object of the invention to overcome or at least mitigate the above mentioned difficulties by providing a process which can be used to prepare immobilized biological materials with high activity and high physical strength by cross-linking with polyazetidine.

STATEMENT OF THE INVENTION

The invention provides a process for immobilizing biological material by cross-linking with polyazetidine, which process comprises the sequential steps of aqueously mixing biological material with polyazetidine, flocculating the resultant mixture, dewatering the flocculated product, sub-dividing the dewatered product and then curing the sub-divided material to obtain a particle form immobilized product. In this process the cross-linking reaction occurs mainly during the final curing.

The particle-form immobilized product prepared according to the invention is well suited for use in packed-bed columns, where it exhibits a low pressure drop. It is also very resistant to abrasion during grinding and is therefore well suited for use in stirred tank reactors.

In contrast to the above-mentioned prior-art process of EP 0 206 687, the polyazetidine polymer is added to the solution or dispersion before dewatering, thereby avoiding the difficult mixing in a pasty state. The amount of cross-linking agent can be reduced compared to EP 0 206 687 because the mixing is more efficient. Although the polyazetidine is added prior to dewatering, very little polyazetidine is lost during dewatering as it binds almost quantitatively to the flocculated material.

DETAILED DESCRIPTION OF THE INVENTION

Biological materials that can be immobilized by the method of this invention include enzymes, cell mass (intact or disrupted cells, viable or non-viable), antibodies and
5 coenzymes. The material is used as an aqueous solution or dispersion which may be purified as desired by conventional techniques. The degree of purification is not critical to the practice of the invention, but influences the properties of the immobilized product.

10 It has been found that the quantity of water present in the reaction mixture is not critical. Excess water will be removed during dewatering without any serious loss of active material. Thus, water may be added to obtain a convenient consistency. Conveniently, the biological material is
15 added in the form of an aqueous dispersion or solution.

The polyazetidine polymer used for the practice of this invention may be any water-soluble polymer with a substantial content of reactive azetidine rings, such as those prepared by reacting a polyamide with epichlorohydrin according to German patent publication DT-AS-1,177,824. Examples
20 of commercially available products are Polycup 2002, Kymene 557H and Reten 304 (these are all trade marks of Hercules Inc., USA).

The amount of polyazetidine may be used to control
25 the properties of the immobilized product. Thus, a high amount generally yields physically strong particles, whereas a low amount yields particles with low diffusion restriction and therefore with high activity. The suitable amount will usually be in the range about 0.1 - about 10% (hereinafter, %
30 are by weight of dry matter in the biological material), typically about 0.5 - about 5%, e.g. about 0.5 to about 3%.

pH throughout the process is generally around neutral, mostly between about 5 and about 9. Higher or lower pH may be preferred depending on enzyme stability. A buffer
35 may be included to stabilize pH during the reaction.

Optionally, glutaraldehyde may be used as a cross-linking agent in addition to polyazetidine. The amount of glutaraldehyde may be up to about 40% (of dry matter, as above), typically below about 15%. If glutaraldehyde is used, it is preferred to hold the mixture of the biological material and glutaraldehyde to let cross-linking occur, e.g. for about 5 minutes to about 1 hour, either before or after the addition of polyazetidine.

Optionally, auxiliary cross-linking agents containing groups that react with polyazetidine, such as -NH_2 groups (e.g. polyethylene imine, chitosan, albumen or gelatine) or -COOH groups (e.g. carboxy-methyl cellulose) may be added to the reaction mixture. If glutaraldehyde is used, any -NH_2 containing agent should preferably be added prior to the glutaraldehyde. These may be used e.g. in order to increase the physical strength, to adjust the consistency of the filter cake for easy handling. Also, in some cases, it may be desirable to include these in order to dilute the activity of the immobilized material, e.g. in the case of penicillin acylase. The amount of auxiliary agent may be up to 100% of dry matter. Preferred quantities are for polyethylene imine up to 20%, for albumen or gelatine up to 50%, and for carboxy-methyl cellulose up to 10%.

Temperature during mixing (and holding, if used) is generally in the range $0\text{--}60^\circ\text{C}$. Temperature near to ambient is often convenient, but lower temperature may be needed due to enzyme instability.

It may be necessary to add a conventional flocculant before dewatering to get satisfactory flocculation. The need for this as well as suitable type and quantity of flocculant are easily determined by someone skilled in the art.

Polyazetidine can act as a cationic flocculant, besides acting as a cross-linking agent. Thus, for negatively charged materials a separate flocculant can be omitted if sufficient polyazetidine is added.

The dewatering step of the invention is intended to remove excess water after flocculation thereby generating a pasty mass. It is conveniently done by filtration or centrifugation.

5 Sub-dividing according to the invention is done prior to curing, as the consistency of the pasty mass at this stage permits easy forming into individual particles of controlled size. A preferred technique is extrusion. Option-
ally, the extruded particles may be rounded (spheronized)
10 before or after curing, e.g. by the "Marumerizer" technique disclosed in British patent specification GB 1,362,265.

The curing step of the invention serves to remove water and to let the cross-linking reaction proceed, so that a physically strong product is obtained. Preferred techniques
15 are air drying or fluidized-bed drying, generally at 15-80°C. In case of very sensitive biological materials, low temperature drying or freeze-drying may be needed.

Preferably, the immobilized product is dried to a water content below around 25% w/w. With an ultimate water
20 content of above around 25% the microbial stability of the product may be unsatisfactory. Furthermore, with water content above around 25% the cross-linking with the polyazetidine prepolymer may not have taken place or may not be complete; the particles may tend to aggregate over time in
25 storage. Drying to a water content of below about 10% may inactivate the biological material.

The process of the invention leads to an immobilized product of high physical strength, and consequently low pressure drop when used in a packed bed. The practical effect
30 is illustrated by the fact that an immobilized product according to the invention (penicillin acylase derived from Fusarium) with a pressure drop value of 2-4 (measured as in Example 1) can be used in a fixed-bed column reactor with a height of 1 meter and a diameter of 1 meter with a holding
35 time of 1 minute. The pressure drop over the bed remains

nearly constant for more than 6 months. A similar prior-art product with a pressure drop value of more than 10 is not able to withstand these conditions.

The high physical strength of a product immobilized according to the invention also results in low grinding loss, as seen in the examples that follow. Products with low grinding loss are significantly more stable in a continuous stirred tank reactor. With penicillin acylase immobilized according to the invention, practical lifetimes of more than 4 months in 4 m³ reactor systems can be expected with activity loss of no more than 50%.

UTILITY

The process of the invention is widely applicable to immobilization of biological materials, such as enzymes, cell mass, coenzymes and antibodies (monoclonal or polyclonal).

Enzymes may be immobilized in the form of enzymatically active cells, of partly or fully homogenized cell paste, or as a largely cell-free enzyme solution. Some examples where the method of the invention is particularly advantageous follow:

- glucose isomerase from Streptomyces sp., e.g. from the following species:

- | | |
|----|---------------------------------|
| 25 | S. murinus
(EP 0 194 760) |
| | S. flavovirens |
| | S. achromogenus |
| | S. echinatus |
| 30 | S. wedmorensis |
| | S. albus
(US 3,616,221) |
| | S. olivochromogenes |
| | S. venezuelae
(US 3,622,463) |

- S. griseoflavus
(US 4,351,903)
- S. glaucescens
(US 4,137,126)
- 5 S. olivaceus
(US 3,625,828)
- S. galbus
S. gracilis
S. matensis
10 S. niveus
S. platensis
(Hungarian patent 12,415)
- S. violaceoniger
(German patent 2,417,642)
- 15 S. acidodurans
(US 4,399,222)
- S. phaeochromogenes
S. fradiae
S. roseochromogenes
20 S. olivaceus
S. californicus
S. vanaceus
S. virginiae
(Japanese patent publication 69-28,473)
- 25 - glucose isomerase from Bacillus coagulans, (see US 3,979,261).
- glucose isomerase from Actinoplanes sp., especially A. missouriensis.
- glucoamylase from Aspergillus sp., especially from black
30 Aspergilli and more especially from A. niger (see US patent 3,677,902), or from Rhizopus sp., especially Rh. delemar or Rh. niveus.

- penicillin-V acylase from Fusarium sp., especially F. anguioides, F. argiilaceum, F. avenaceum, F. bulbigenum, F. coeruleum, F. culmorum, F. equiseti, F. lateritium, F. minimum, F. monoliforme, F. oxysporum, F. sambucinum, F. semisectum, F. solani, and F. sulphureum (see GB 891,173).
5
- penicillin acylase from Eschericia coli, Proteus rettgeri, Kluyvera citrophila, Bacillus sphaericus, Bovista plumbea or Bacillus megaterium.
- lactase from Kluyveromyces sp., especially from K. fragilis
10 or K. lactis.
- cyanide hydratase according to Danish application DK 87/1283.
- nitrilase, nitrile hydratase or amidase, especially from Rhodococcus sp., Pseudomonas sp. or Brevibacterium sp. See
15 US 4,001,081, EP 0 093 782 and EP 0 188 316.

Cell mass to be immobilized by the process of the invention may be in the form of viable or non-viable intact cells as well as homogenized cell paste. The cells are preferably of microbial or plant origin. Some preferred examples
20 follow:

- viable cells for use in bioconversion, e.g. yeast for ethanol fermentation.
- cells with enzymatic activity, e.g. fungal mycelium containing cyanide hydratase, see EP 0 061 249 and EP 0 116
25 423.
- cell mass for use in adsorptive removal, e.g. of heavy metals, see EP 0 181 497, US 4,320,093, US 4,298,334, US 4,293,333 and JP-A 49-104,454.

EXAMPLE 1

Glucose isomerase containing cells were produced by fermentation of Streptomyces murinus, strain DSM 3253, according to European patent publication EP 0 194 760.

- 5 The cells were harvested by centrifugation of the culture broth. The cell sludge had a dry substance content of 7.0%.

The general immobilization procedure was as follows;

- 10 To 300 g cell sludge was added 300 g deionized water containing 1.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. pH was adjusted to 7.5. The indicated amount of polyethyleneimine (Sedipur, product of BASF, West Germany) was added, and after thorough mixing the mixture was cross-linked by addition of 15% active glutar-
15 aldehyde based on cell sludge dry substance plus polyethyleneimine dry substance. After 1 hour the polyazetidine (Polycup® 2002) was added and thoroughly mixed with the cross-linked cell suspension.

- The mixture was then flocculated by addition of a
20 cationic flocculant, Superfloc C521 (Cyanamid Int.). The cross-linked enzyme was recovered by filtration, formed into particles by extrusion through a 0.8 mm screen and dried at room temperature.

- The glucose isomerase activity was measured by NOVO
25 Analysis Method F-855310 (available on request from Novo Industri A/S, Denmark) and the physical stability determined as pressure drop over a column.

The pressure drop was measured over a column with a diameter of 24 mm and an enzyme bed height of 4 cm (5 g

enzyme). The solution, 45% glucose in demineralized water with 1 g MgSO_4 /l, was pumped through the column at a rate of 40 g/min at 60°C. The pressure drop (in mm of liquid) describes the physical stability of the enzyme particle, i.e. a low pressure drop corresponds to a good physical stability. The results are shown in the table below.

		Glucose isomerase activity ($\mu\text{mol}/\text{min}/\text{g}$)	Pressure drop (mm)
10	0% poly-azetidine	614	400
5% poly-ethylene-imine	2.5% poly-azetidine	667	99
15	5% poly-azetidine	586	57
20	0% poly-azetidine	692	105
10% poly-ethylene-imine	2.5% poly-azetidine	578	11
25	5% poly-azetidine	506	10

Preparations with pressure drops exceeding about 20 mm liquid are considered unsuitable for industrial glucose isomerase columns, and thus the polyazetidine is essential to make an industrially useful enzyme in this example. Activity decreases slightly with increasing polyazetidine concentration.

This example clearly shows the improvement of physical stability of the immobilized preparations obtained with polyazetidine.

EXAMPLE 2

A similar experiment to that described in Example 1 was performed using a higher yielding descendant of DSM 3253.

The dry substance content of the cell sludge was 5.3%, and the cells were partially disrupted by homogenization. Otherwise the immobilization was performed as described in Example 1.

The results are given in the table below.

		Glucose isomerase activity ($\mu\text{mol}/\text{min}/\text{g}$)	Pressure drop (mm)
10	5% poly-ethylene-imine	0% poly-azetidine	556
		1% poly-azetidine	855
		2.5% poly-azetidine	839
		5% poly-azetidine	707
25	10% poly-ethylene-imine	0% poly-azetidine	651
		1% poly-azetidine	975
		2.5% poly-azetidine	911
		5% poly-azetidine	853
30	10% poly-ethylene-imine	0% poly-azetidine	12
		1% poly-azetidine	10
		2.5% poly-azetidine	5
		5% poly-azetidine	3

From these experiments, it can be concluded that the polyazetidine improves the physical stability of even very physically stable formulations.